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Uterine cervix nitric oxide and human papillomavirus infection in women

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I Rahkola P, Mikkola TS, Nieminen P, Ylikorkala O, Väisänen-Tommiska M. Abnormal cervical cytology is associated with increased nitric oxide release in the uterine cervix.
Acta Obstetricia Gynecologica Scandinavica 2009; 88:417-421.

II Rahkola P, Mikkola TS, Ylikorkala O, Väisänen-Tommiska M. Association between high-risk papillomavirus DNA and nitric oxide release in the human uterine cervix.
Gynecology Oncology 2009; 114:323-326.

III Rahkola P, Väisänen-Tommiska M, Hiltunen-Back E, Auvinen E, Ylikorkala O, Mikkola TS. Cervical nitric oxide release in *Chlamydia trachomatis* and high-risk human papillomavirus infection. Acta Obstetricia Gynecologica Scandinavica, 2011; 90:961-965.

IV Rahkola-Soisalo P[a], Savolainen-Peltonen H[a], Väisänen-Tommiska M, Butzow R, Ylikorkala O, Mikkola TS. High-risk human papillomavirus-induced expression of endothelial and inducible nitric oxide synthase in human uterine cervix. Submitted.
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V Rahkola P, Väisänen-Tommiska M, Tuomikoski P, Ylikorkala O, Mikkola TS. Cervical nitric oxide release and persistence of high-risk human papillomavirus in women.
International Journal of Cancer 2011; 128:2933-2937.

 In addition, some unpublished data are included.

ABBREVIATIONS

ASC-US	atypical squamous cells with undetermined significance
ASC-H	atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion
CI	confidence interval
CIN	cervical intraepithelial neoplasia
eNOS	endothelial nitric oxide synthase
HPV	human papillomavirus
HSIL	high-grade squamous intraepithelial lesion
iNOS	inducible nitric oxide synthase
LSIL	low-grade squamous epithelial lesion
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NOx	nitric oxide metabolites, nitrate and nitrite
NS	nonsignificant
OR	odds ratio
SE	standard error

ABSTRACT

The human uterine cervix produces nitric oxide, which participates in the regulation of immunological reactions. Nitric oxide also appears to play a role in carcinogenesis and in the progression/regression of precancerous lesions. The cervix is a target for human papillomavirus (HPV) infection, the most common genital infection, and this virus is necessary in the connection with precancerous and cancerous lesions of the cervical epithelium. Local factors, such as nitric oxide, may have a role in defense against HPV infection and/or in the results of HPV-induced cellular changes.

The levels of nitric oxide measured by those of its metabolites (NOx) in cervical fluid were compared in women with and without HPV, and different cytological and histological changes. Altogether, 801 women on 1033 occasions were studied. The effects of other relevant gynecological infections on cervical NOx levels were assessed independently and in association with high-risk HPV. Moreover, the expression of nitric oxide-producing endothelial (e) and inducible (i) nitric oxide synthase (NOS) in cervical epithelium was determined by Western blotting and immunohistochemistry.

In women with cytology suggestive of HPV infection, the levels of cervical fluid NOx were twofold greater than in women with normal cytology. A similar difference was seen in NOx levels between women with (median 47.1 μmol/l) and without high-risk HPV infection (median 23.8 μmol/l). Cervical dysplastic lesions were associated with elevated levels of cervical fluid NOx; the women with low-grade lesions appeared to have the highest cervical nitric oxide production.

Chlamydia trachomatis infection was associated with elevated NOx levels. Neither bacterial vaginosis nor candida infection affected cervical fluid NOx levels, but the number of women with these infections was limited.

Cervical expression levels of eNOS and iNOS were higher in women with high-risk HPV infection compared with those without. Endothelial NOS was localized to vascular endothelium, while iNOS was mainly detected in the basal layer of squamous epithelial cells.

To evaluate the clinical significance of these findings, high-risk HPV-infected women without any treatment were followed for 12 months. At baseline, cervical fluid NOx levels were higher in women with persistent high-risk HPV infection compared with those with eradicated high-risk HPV infection. Baseline NOx levels over the 75th percentile (87 μmol/l) predicted high-risk HPV persistence (odds ratio 4.1). This NOx cut-off value showed 33% sensitivity and 90% specificity for prediction of high-risk HPV persistence.

In conclusion, HPV infection was related with elevated levels of cervical fluid NOx as a result of activated expression of eNOS and iNOS proteins. High cervical fluid NOx levels were associated with high-risk HPV persistence, though the low sensitivity of this test to identify women with persistent high-risk HPV limits its clinical use.

INTRODUCTION

It has been estimated that infection, directly or mediated through inflammation, could explain approximately 20% of all cancers (Aggarwal *et al*. 2006). This is certainly true for uterine cervical cancer, which, worldwide after breast cancer, is the second most common cancer in women (Jemal *et al*. 2011). It has now been conclusively documented that this form of cancer does not arise without persistent HPV infection (zur Hausen 2002, Bosch *et al*. 2007). It is interesting that although most women acquire genital HPV infection during their lifetimes (Ebrahim *et al*. 2005, Moscicki 2005), only a few are diagnosed with premalignant lesions or ultimately cervical cancer as their immunological defense fails to eradicate this infection (Stanley 2009). For the moment it is not known which HPV infections persist and lead to cervical cancer and that is why expensive and laborious follow-up of patients is needed. Furthermore, cervical lesions are commonly treated by means of excision, which increases the risk of premature labor (Jakobsson *et al*. 2007). Conization in many cases also represents overtreatment of possibly spontaneously regressing dysplastic lesions. Vaccination against the most common high-risk HPV types has been developed (Christensen *et al.* 1990, Zhou *et al*. 1991, Koutsky *et al*. 2002), but this approach is expensive and laborious. Moreover, it has been postulated that other HPV types that are not covered by vaccination may become associated with cervical cancer when the currently known high-risk HPVs are eradicated (Chan *et al*. 2009, Merikukka *et al.* 2011). Therefore, it is very important to find markers that help to identify HPV-infected women at risk of progression of cervical lesions and ultimately cancer.

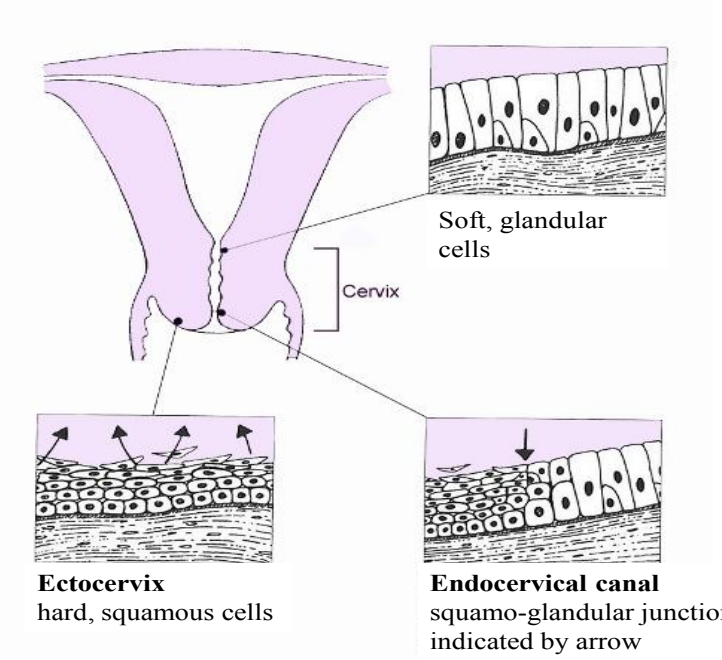
An "endothelium relaxing agent" was described in 1987 (Ignarro *et al.* 1987, Palmer *et al.* 1987). Subsequently it was shown to be nitric oxide, an uncharged gas molecule which is released locally at the site of the synthesis (Alderton *et al.* 2001, Crane *et al.* 2010). Nitric oxide has proven to be significant in various physiological and pathophysiological conditions (Bogdan 2001, Lala *et al.* 2001, Kolluru *et al.* 2010). It is produced by cervical cells (Väisänen-Tommiska *et al*. 2006b) and therefore it is possible that cervical nitric oxide could affect HPV infection. The present study was designed to elucidate this association in women. Moreover, cervical nitric oxide levels were assessed in women infected with *Chlamydia trachomatis*, which is an independent risk factor of cervical cancer (Madeleine *et al*. 2007).

REVIEW OF THE LITERATURE

Uterine Cervix

The human uterine cervix has to have unique properties, since it must stay rigid during pregnancy but open during labor (Nuutila 1999). It is composed of an extracellular matrix consisting predominantly of collagens with elastin and proteoglycans, and a cellular part containing smooth muscle cells, fibroblasts, epithelium and blood vessels (Ludmir *et al*. 2000). Multilayered squamous epithelial cells line the cervix in its lower part (ectocervix), while a single layer of mucus-secreting glandular epithelial cells line the upper part (endocervix) (Roos *et al.* 2006) (Figure 1). The transformation zone is the area in which glandular epithelium changes progressively into squamous epithelium (Delvenne *et al.* 2007), and metaplastic cells at this squamo-glandular junction are vulnerable to various infections (Burd 2003, Delvenne *et al.* 2007). The basal cells, the only ones dividing actively, migrate upward and differentiate to keratinocytes, which, after reaching the surface of the epithelium, die through apoptosis (Hebner *et al.* 2006).

Figure 1. A schematic illustration of components present in the human uterine cervix (with permission of the copyright holder. Szarewski A 2007, Altman Publishing, UK).



Cervical immune defense consists of a barrier of mucus covering epithelial cells (Johansson *et al.* 2003). These epithelial cells, together with endothelial and inflammatory cells, serve as antigen-presenting cells and produce mediators such as cytokines and interleukins that further increase inflammation. The local cervical immune response is also influenced by sex hormones which regulate immunoglobulin A antibody responses (Johansson *et al.* 2003). Several cervical cells, such as squamous and glandular epithelial, hematopoietic, and stromal cells are also capable of producing nitric oxide (Agarwal *et al*. 2005), a free radical gas involved in cervical ripening during pregnancy (Väisänen-Tommiska 2006a).

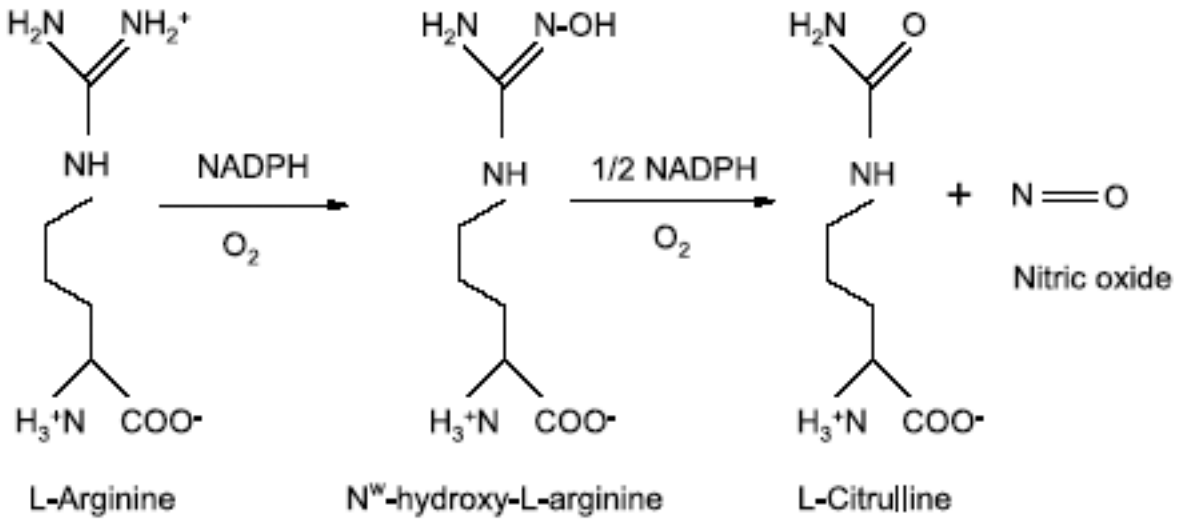
Nitric oxide

An "endothelium relaxing agent" was described in 1987 (Ignarro *et al.* 1987, Palmer *et al.* 1987). It was soon shown that this "new" agent is detected in almost all cell types and that it has a role, for example, in immunology and carcinogenesis (Bogdan 2001, Coleman 2001, Lala *et al.* 2001, Korhonen *et al.* 2005, Ridnour *et al.* 2006). Nitric oxide acts as an intra- and intercellular messenger and it is highly diffusible both in aqueous and hydrophobic environments (Thomas *et al*. 2008).

Biosynthesis

Nitric oxide is generated from the amino acid L-arginine and molecular oxygen by nitric oxide synthase (NOS) (Alderton *et al*. 2001, Crane *et al*. 2010). This biosynthesis consists of two-stage oxidation of L-arginine to L-citrulline and nitric oxide via an intermediate, N^{w}-hydroxy-L-arginine (Figure 2). Adenine dinucleotide phosphate works as a cosubstrate and heme, tetrahydrobiopterin, calmodulin and flavine-adenine mononucleotide as co-factors in this synthesis (Alderton *et al*. 2001, Crane *et al.* 2010).

Figure 2. Biosynthesis of nitric oxide.



Three NOS isoenzymes have been detected: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) (Pollock *et al*. 1991, Xie *et al.* 1992, Nakane *et al.* 1993). Of constitutive NOSs, nNOS is expressed mainly in neurons (Garthwaite 2008) while eNOS is expressed particularly in the vascular system (Shaul 2002). These NOSs are activated by intracellular calcium and they produce nitric oxide in nanomolar concentrations (Alderton *et al*. 2001, Shaul 2002). The third isoform, iNOS, is calcium-independent and is activated by inflammation (Alderton *et al*. 2001, Bogdan 2001, Aktan 2004). Activation of iNOS results in nitric oxide concentrations in the nanomolar to micromolar range (Espey *et al*. 2000). Inducible NOS is detected in various cells, such as monocytes and macrophages (Xie et al. 1992, Thomassen *et al.* 2001), natural killer cells, endothelial and epithelial cells, and keratinocytes (Langrehr *et al.* 1993, Bogdan *et al.* 2000).

Table 1. Characteristics of endothelial and inducible nitric oxide synthase (NOS) that are important in the human uterine cervix.

	Endothelial NOS	**Inducible NOS**
Molecular weight	140 kDa	130 kDa
Activation	Calcium - calmodulin	Transcription
Magnitude of nitric oxide concentration	Nanomolar levels (10^{-9})	Micromolar levels (10^{-6})
Site of primary expression	Endothelial cells	Macrophages, epithelial cells
Regulators	Acetylcholine Bradykinin Sex hormones Physical exercise	Inflammatory mediators Cytokines Lipopolysaccharide Prostaglandins Viral components

Assessment

Direct measurement of nitric oxide is difficult because of its short half-life (3-5 s) and rapid interactions with various organic molecules (Hong *et al*. 2009), although several techniques, such as chemiluminescence, fluorometry and electrochemical methods have been employed, especially in scientific work (Nagano *et al*. 2002). Nitric oxide has also been assessed by optical means, electron paramagnetic resonance, magnetic resonance, and positron emission tomography, but these technically complex methods are not useful in clinical research (Hong *et al*. 2009).

The Griess reaction is one of the most widely used indirect methods of nitric oxide detection. This colorimetric method is based on the rapid conversion of nitric oxide to its stable intermediates nitrate and nitrite (NOx). Nitrate in the sample is first reduced to nitrite (Green *et al*. 1982, Miranda *et al.* 2001). Thereafter, a two-step diazotization reaction produces a diazonium ion coupled to N-(1-naphthyl)ethylenediamine to form an azo product that absorbs

light at a 540nm wavelength (Miranda *et al.* 2001, Väisänen-Tommiska *et al*. 2003, Bryan *et al*. 2007). Food rich in nitrate elevates plasma NOx levels (Jungersten *et al.* 1996) and gastrointestinal microbes are capable of producing nitric oxide (Sobko *et al*. 2006). Thus, if nitric oxide production is detected in circulating blood, oral intake of nitrate/nitrite-containing food must be restricted. However, cervical fluid NOx levels are not influenced by food or gastrointestinal nitric oxide (Väisänen-Tommiska *et al*. 2003) and therefore the Griess reaction is a suitable method to indirectly assess nitric oxide production in the cervix.

Different tissue NOS isoforms can be located by Western blotting and immunohistochemistry (Tornblom *et al.* 2005, Väisänen-Tommiska *et al*. 2006b). Nitric oxide synthase proteins can be measured quantitatively by Western blotting. Semi-quantitatively, NOSs can be evaluated by immunohistochemistry and this method enables localization of cells producing NOSs.

Mechanisms of action

Nitric oxide reacts with various molecules and proteins either directly or indirectly (Bogdan 2001, Thomas *et al*. 2008). It can directly react with a target molecule, bringing about, for example nitrosylation of a cysteine residue, which inhibits protein synthesis. Indirect reactions encompass non-enzymatic oxidation of nitric oxide to reactive nitrogen species, which thereafter react with target molecules (Bogdan 2001, Korhonen, *et al.* 2005, Thomas *et al.* 2008). These various reaction mechanisms and numerous target molecules of nitric oxide explain its diverse and often opposing biological effects, such as its capability to both promote and inhibit inflammatory and carcinogenic responses (Bogdan 2001, Coleman 2001, Korhonen *et al*. 2005, Ridnour *et al.* 2006, Thomas *et al.* 2008).

The effects of nitric oxide in

The nervous system and vasculature

Nitric oxide via nNOS takes part in inter- and intraneuronal signaling both in the brain and in the peripheral nervous system (Zhou *et al*. 2009, Steinert *et al.* 2010). It mediates synaptic plasticity and takes part in regulation of respiratory and circadian rhythms. It also participates in learning and memory. Neuronal nitric oxide dysfunction may be neurotoxic and lead to neurodegenerative disorders, such as Alzheimer's disease, multiple sclerosis and Parkinson's

disease (Zhou *et al*. 2009, Steinert *et al.* 2010).

Nitric oxide via eNOS is an important vasoactive compound (Chatterjee *et al*. 2008, Kolluru *et al*. 2010). It regulates vascular tone, especially vasodilatation. It also mediates platelet function and has an important role in endothelial cell migration and proliferation, and angiogenesis. Dysfunction of eNOS has been related to occlusive cardiovascular diseases such as hypertension, peripheral vascular disease, stroke and coronary heart disease (Chatterjee, *et al*. 2008, Kolluru *et al*. 2010).

Immunology

Both eNOS and iNOS are involved in immunology, though iNOS is the most studied in this regard (Bogdan 2001, Coleman 2001, Ridnour *et al.* 2006). Various pro-inflammatory mediators induce iNOS activation indirectly (Drapier *et al*. 1988, Xie *et al.* 1993, Korhonen *et al*. 2005), but viral components or viral replication may also activate iNOS directly (Hori *et al*. 1999, Akaike *et al.* 2000). The triggering mechanisms of eNOS are unclear, but they may be linked to activated neutrophils (Rabelink *et al.* 2006).

Nitric oxide can function as a toxic agent and eradicate infectious organisms, for example by inhibiting virus replication. It may regulate both innate and adaptive immunology by affecting the production of inflammation-related cytokines such as interleukins and interferons. In addition, nitric oxide modulates the function of T- and B-lymphocytes (Akaike *et al*. 2000, Bogdan 2001, Coleman 2001, Zaki *et al*. 2005).

Nitric oxide may eradicate infectious organisms either directly or indirectly. Direct antimicrobial activity encompasses, for example, DNA mutations of pathogens, inhibition of protein synthesis, and enzyme activation (Bogdan 2001). Indirect reactions involve the formation of reactive nitrogen species (Wink *et al.* 1996) and interaction with reactive oxygen species (Bogdan 2001, Coleman 2001, Korhonen *et al.* 2005). Reactive oxygen species are produced in monocytes and macrophages during infection (Badwey *et al*. 1980) and their consequent reactions with nitric oxide lead to the formation of even more reactive radicals, such as peroxynitrate (Bogdan et al. 2000, Coleman 2001). These reactions, however, are non-selective and may also be harmful to the host cells, as seen in pneumonia caused by influenza virus, in which alveolar exudates as well as destruction of pulmonary architecture

are increased in iNOS$^+$ mice. Furthermore, the effects of nitric oxide are not always lethal, but they may cause viral mutations which may lead to the generation of new resistant strains (Zaki *et al*. 2005). Thus, even if nitric oxide eradicates infectious agents, this may turn deleterious if toxic effects are targeted toward host cells, especially in conditions of chronic inflammation (Sawa *et al.* 2006).

Cancer

Evidence from *in vitro* and animal studies shows that nitric oxide may both promote and suppress cancer (Goodman *et al*. 2004, Ridnour *et al*. 2006). Both eNOS and iNOS are expressed by stromal as well as tumor cells (Fukumura *et al.* 2006, Ying *et al*. 2007). The role of nitric oxide depends on several factors, such as local microenvironment, and most importantly, on concentration; low levels favor while high ones suppress cancer (Goodman *et al.* 2004, Ridnour *et al*. 2006). Nitric oxide can promote cancer by directly damaging DNA and inactivating DNA-repair proteins (Lala *et al*. 2001, Fukumura *et al*. 2006). In addition, nitric oxide can favor tumor cell migration, invasion and angiogenesis. This may take place either directly or indirectly via modulation of other factors, such as vascular endothelial growth factor and prostaglandins (Lala *et al.* 2001, Fukumura *et al.* 2006, Ridnour *et al*. 2006). On the other hand, nitric oxide may also suppress cancer, since it is capable of inducing tumor cell apoptosis (Fukumura *et al.* 2006). In fact, it has been shown that tumor growth progresses if the host fails to sustain nitric oxide concentrations high enough to bring about tumor cell apoptosis (Heller 2008). Thus, nitric oxide may have a dual effect on cancer and its role may change from tumor suppressor to promoter during the course of a long cancer pathway (Ridnour *et al*. 2006).

Human papillomavirus infection

Human papillomaviruses (HPVs) are double-stranded DNA viruses with eight genes encoding six early proteins and two capsid proteins (Baker *et al.* 1991). About 150 different HPV types have been recognized and more genotypes are being detected (zur Hausen 1996, Stanley *et al*. 2007). Roughly 40 HPV types are capable of infecting the genital mucosa. The infection can be subclinical (Moscicki 2005, Trottier *et al*. 2006a) or manifest with genital warts, vulvar,

vaginal and cervical intraepithelial neoplasia, and ultimately cancer (Parkin *et al*. 2006, Insinga *et al*. 2008). Human papillomaviruses can be divided into low-risk and high-risk HPV genotypes depending on their oncogenic potential (Bosch, *et al* 2003). Low-risk HPVs cause benign genital warts and low-grade dysplastic lesions, while high-risk HPVs can cause both low- and high-grade vulvar, vaginal and cervical lesions (Munoz *et al.* 2003, Clifford *et al*. 2006). It has been suggested that cervical cancer does not arise without the presence of persistent high-risk HPV infection (Bosch *et al.* 2002, zur Hausen 2002). High-risk HPV 16 and 18 are the most common genotypes found in all genital HPV-related malignancies (Stanley 2010a).

Detection

In cervical cells HPV induces typical cytological changes such as koilocytosis and dyskeratosis which can be detected in Pap smears (Purola *et al*. 1977). The sensitivity of Pap smears to revel lesions similar to or more severe than CIN 2 (cervical intraepithelial neoplasia grade 2) is lower compared with HPV tests (Arbyn *et al*. 2006) and thus the use of HPV tests has become more popular (Schiffman *et al.* 2011). Human papillomavirus tests can detect HPV viral DNA or RNA (Sandri *et al.* 2006, Dockter *et al*. 2009). DNA-based tests detect the presence of the HPV virus genome, whereas RNA tests detect the expression of messenger RNA of oncogenes E6 and E7. Two of the most common DNA-based HPV tests are the polymerase chain reaction-utilizing target amplification method (Roche Amplicor, Branchburg, NY, USA) and the hybrid capture method using signal amplification (Digene Corporation, Gaithesburg, MD) (Iftner *et al*. 2003). These both tests detect the same 13 high-risk HPV genotypes (Sandri *et al.* 2006, Carozzi *et al.* 2007).

Epidemiology

Human papillomavirus infection is the most common genital infection. In sexually active women the lifetime risk of infection is 80% (Ebrahim *et al*. 2005, Moscicki 2005). The incidence is highest after the initiation of sexual life (Moscicki *et al.* 2001, Kahn *et al.* 2002) and another incidence peak is seen in postmenopausal women (Burchell *et al.* 2006), the latter probably representing reactivation of an earlier infection (Burchell *et al.* 2006, Trottier *et al*.

2006b). The prevalence of HPV infection among women ranges from 2 to 40% (Bosch *et al.* 2003, Baseman *et al.* 2005). In a Finnish cohort the prevalence of HPV was found to be 33% among first-year University students (Auvinen *et al.* 2005). Biological factors such as inadequate production of cervical mucus, and abundant glandular and metaplastic epithelium make young women more susceptible to HPV (Kahn *et al.* 2002, Moscicki 2005, Burchell *et al.* 2006, Delvenne *et al*. 2007).

Natural course and immunology of human papillomavirus infection

Most HPV infections are transient and 90% of women without any immune compromise eradicate the virus within 2 years (Moscicki *et al.* 2004, Stanley 2010b, Woo *et al*. 2010). Increasing age (Ho *et al.* 1998), high virus load (Maucort-Boulch *et al*. 2010), infection with HPV 16 (Richardson *et al*. 2003) or with multiple HPV types (Ho *et al*. 1998, Nielsen *et al.* 2010), and smoking (Matsumoto *et al.* 2010) are associated with longer clearance time. Moreover, some women are genetically more vulnerable to HPV persistence (Wang *et al.* 2009, Ferguson *et al*. 2011).

Human papillomavirus infection is initiated from the basal layer of squamous cervical cells in which the number of viral copies is kept low (Burd 2003, Hebner *et al.* 2006). The low number of viral copies helps HPV to avoid the host immune system. Human papillomavirus is incapable of amplifying its DNA and thus it needs host cell DNA machinery. Viral early proteins take part in this genome replication. The expression of early proteins E6 and E7, also called as oncoproteins, delays normal cell maturation and arrests cervical cell apoptosis, which leads to immortalizing of infected cells (Burd 2003, Hebner *et al.* 2006). At the surface of the epithelium the number of viral copies is high and capsid proteins L1 and L2 are synthesized. They are needed for viral genome encapsulation and finally new virus particles are shed to initiate a new infectious cycle (Woodman *et al.* 2007).

The local immune system in the cervix is important for HPV clearance (Stanley 2006, Song *et al*. 2008). This clearance is associated with T-helper-1-type cytokines (Stanley 2006, Song *et al.* 2008, Syrjänen *et al.* 2009), including interferon-γ and tumor necrosis factor-α, whereas T-helper-2 cytokines such as interleukin-10 and interleukin-13 are inhibitory as regards cell-mediated responses (Spellberg *et al*. 2001) and thus for virus eradication (Stanley 2006). Cleared HPV infection is associated with humoral antibodies against the infected HPV

genotype, but these antibodies are not protective against other HPV genotypes (Palmroth *et al.* 2010).

Cellular and dysplastic changes associated with human papillomavirus infection

At the cellular level, HPV DNA exists typically in an episomal form in low-grade lesions, whereas integration into the host genome increases in parallel with lesion grade (Figure 3) (Munger *et al*. 2004, Woodman *et al*. 2007). High-grade lesions are associated with high expression of oncogenes E6 and E7, which favors high-risk HPV persistence, a key factor in neoplastic transformation of cervical cells (Burd 2003, Munger *et al*. 2004, Hebner *et al.* 2006).

Approximately 66–75% of women with detectable high-risk HPV infection do not have any cytological abnormalities, and only a small proportion of women are diagnosed with dysplastic lesions (Schiffman *et al.* 2011) (Figure 3). Squamous cell lesions are histologically divided into CIN grades 1, 2 and 3 (Kumar *et al.* 2010). The cellular hallmarks involve atypical cell proliferation with altered size, shape, and polarity, an increased number of mitoses and disturbed epithelial architecture. In CIN 1 lesions morphological changes are mild and restricted to the lowest 1/3 of the epithelium, whereas in CIN 2 lesions the changes extend to 2/3 of the epithelium, and in CIN 3 the entire epithelial thickness is involved. Cancer arises when the basal membrane is breached and infiltrative growth or metastases exist (Kumar *et al.* 2010). Adenocarcinoma (and its precursors) arises from cervical glandular cells and it accounts worldwide for 10–20% (Castellsague *et al*. 2008) and in Finland 30% (Finnish Cancer Registry) of all cervical cancers. In contrast to squamous epithelial cancer, the incidence of adenocarcinoma is increasing (Dahlström *et al*. 2010).

Of low-grade lesions only some are caused by high-risk HPV, whereas practically all high-grade lesions are accompanied by the presence of high-risk HPV (Table 2). The time between high-risk HPV infection and the development of CIN 3 is 3–5 years, but the progression to invasive cancer takes a further 10–20 years (Stanley 2010b).

Figure 3. Different stages of human papillomavirus-related cervical lesions. The arrows show the direction of viral replication from basal to upper layers of the epithelium. New viruses are shed and they initiate a new infection cycle. (With a permission of the copyright holder, Woodman, *et al.* 2007).

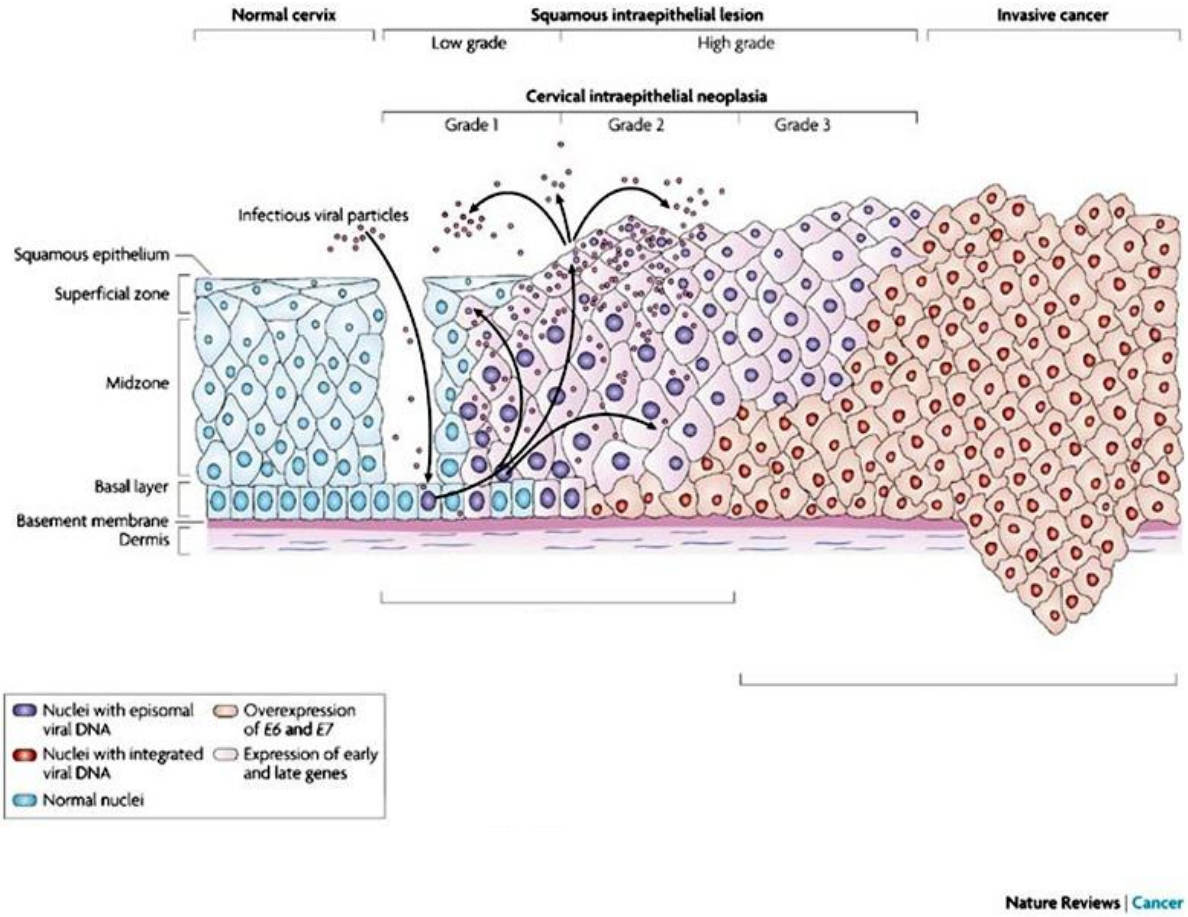


Table 2. Proportion of high-risk human papillomavirus (HPV) infections in connection with different cytological changes and cervical intraepithelial neoplasias (CIN).

	Cytology				Histology		
	Normal	**ASC-US**	**LSIL**	**HSIL**	**CIN 1**	**CIN 2**	**≥ CIN 3**
Percentage of high-risk HPV infected	10[1]	32-78[2,3]	29-92[2,3]	75-97[2,3]	50-88[4,5]	70-90[4,5]	98.8-100[5]

([1]de Sanjose *et al*. 2007, [2] Evans *et al*. 2006, [3]Clifford *et al*. 2006, [4]Castellsague 2008, [5]Castle *et al*. 2010).

ASC-US: atypical squamous cells of undetermined significance
LSIL: low-grade squamous intraepithelial neoplasia
HSIL: high-grade squamous intraepithelial neoplasia

All cervical lesions are dynamic and may regress, persist, or progress (Jordan *et al.* 2009). Of CIN 1 lesions over 90% regress spontaneously (Moscicki *et al.* 2004) and a risk for cervical cancer is estimated to be as high as 1.9% (Pretorius *et al.* 2006). As lesion severity increases the tendency to regress decreases. It has been evaluated that 43% of CIN 2 lesions and 32% of CIN 3 lesions regress spontaneously (Ostör *et al.* 1993). However, in young women these numbers are much higher, since 68% of women of < 24 years of age experience resolution of CIN 2 lesion within 3 years (Moscicki *et al.* 2010). High-grade lesions are always treated nowadays and thus it is impossible to assess the true cancer risk associated with these lesions. In an untreated cohort 31.3% of women with CIN 3 lesions developed cervical cancer within 30 years (McCredie *et al.* 2008).

Cervical lesions are detected by colposcopy and directed biopsies and the most common treatment modality is Loop-conization, in which a part of the cervix is removed. This leads to activation of the immune system which results in HPV eradication in almost all cases within one year (Kim *et al.* 2009).

Other relevant gynecological infections

Chlamydia trachomatis

Chlamydia trachomatis is the most common sexually transmitted bacterium (Manavi 2006). Diagnosis of *Chlamydia trachomatis* infection is currently based on detection of its ribosomal RNA in the cervix or urine. The infection may be clinically asymptomatic or cause ascending pelvic inflammatory disease (Manavi 2006). Women infected with *Chlamydia trachomatis* in 20–60% of cases are co-infected with HPV (Denks *et al.* 2007, Keegan *et al.* 2009, Verteramo *et al.* 2009), and though *Chlamydia trachomatis* infection is an independent risk factor of cervical cancer (Paavonen *et al.* 1999, Anttila *et al.* 2001, Smith *et al.* 2002b) it may also serve as a cofactor of high-risk HPV-induced carcinogenesis (Samoff *et al*. 2005).

Bacterial vaginosis

Bacterial vaginosis is a common genital infection in women of reproductive age. The prevalence varies between 8–23% (Marrazzo 2011). Overgrowth of *Gardnerella vaginalis, Mycoplasma hominis*, anaerobic bacteria, and a lack of normal lactobacilli flora, are characteristics of bacterial vaginosis (Tokyol *et al*. 2004, Fitzhugh *et al.* 2008). Diagnosis is based on the presence of a milky homogeneous vaginal discharge with pH a higher than 4.5, a fishy odor after the addition of potassium hydroxide, and the presence of clue cells in Pap smears (Amsel *et al.* 1983, Davis *et al.* 1999). Clue cells are squamous cells that are completely covered by coccobacilli. Despite bacterial overgrowth, bacterial vaginosis is not accompanied by neutrophils (Fitzhugh *et al*. 2008). Spontaneous clearing as well as recurrent infections are common (Marrazzo 2011). Bacterial vaginosis increases the risk of preterm birth (Kurki *et al*. 1992, Kekki *et al.* 2001). It also enhances the risk of acquiring HIV infection (Thurman *et al*. 2010), but probably not HPV (Nam *et al*. 2009) and thus the association of bacterial vaginosis with cervical changes is controversial (Boyle *et al.* 2003, Nam *et al*. 2009).

Candida

Candida is a common cause of vaginitis, with prevalence of 10% (Spence *et al*. 2007) and of which *Candida albicans* accounts for 85–90% of cases (Walker *et al.* 2000). Clinical symptoms are abnormal vaginal discharge and itching, but many women are asymptomatic (Sobel 2007, Spence *et al.* 2007, Fitzhugh *et al*. 2008). Diagnosis can be carried out microscopically by observing the presence of fungal organisms in wet mounts or by detecting yeast forms with long pseudohyphae in Pap smears (Audisio *et al.* 2001, Fitzhugh *et al.* 2008). Candida is not an independent risk factor of high-grade cervical abnormality (Roeters *et al*. 2010). However, women with concomitant candida and ASC-US in Pap smears more often have high-risk HPV compared with women with ASC-US only (Hall *et al.* 2009).

AIMS OF THE STUDY

The present study was undertaken to evaluate the possible involvement of cervical nitric oxide release in cases of HPV infection (Figure 4).

Specific aims were to compare
cervical fluid nitric oxide metabolite levels in women with and without

1. typical HPV-induced cytological abnormalities

2. high-risk HPV

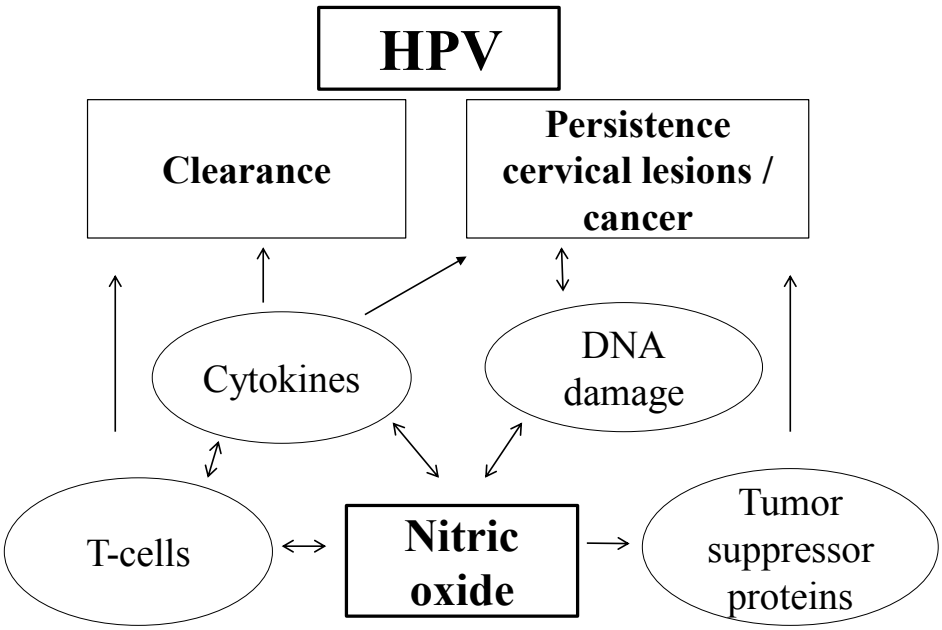
3. high-risk HPV-associated cervical lesions

4. *Chlamydia trachomatis*, bacterial vaginosis and candida infection

5. The expression of eNOS and iNOS in the cervices of women with and without high-risk HPV

6. The cervical fluid nitric oxide metabolite levels in women with persistent versus regressed high-risk HPV

Figure 4. Possible associations between nitric oxide and human papillomavirus (HPV) infection.



SUBJECTS AND METHODS

Subjects

The study was conducted at Helsinki University Central Hospital and approved by the Ethics Committee of the Department of Obstetrics and Gynecology. Altogether, 801 women on 1033 occasions were studied during the period of 2006–2009 (Table 3). Some women took part in both Studies II and V. The women either participated in a screening program or visited outpatient departments of obstetrics and gynecology, or clinics of sexually transmitted diseases. The presence of cervical high-risk HPV was assessed in 504 women (Studies II–V). Pregnant women, women under 18 years of age (Studies I-V), and women with cervical bleeding were excluded from the study (Studies I–III, V). Loop conization was a contraindication for predictive analyses. During the study period all lesions of ≥ CIN 1 were treated in women over 30 years of age and in women < 30 years of age CIN 1 lesions were predominantly followed without treatment.

Table 3. Clinical characteristics of the women studied.

Study	I	II	III	IV	V
Number of women	297	328	39	86	283
Age, mean ± SE Range	36.9 ± 11.8 18–61	35.2 ± 11.4 18–69	27.6 ± 1.1 18–47	38.2 ±1.0 18–58	33.4 ± 11.0 18–64
Nulliparous	152 (51%)	168 (51%)	30 (77%)	33 (38%)	128 (45%)
Use of oral contraceptive	68 (23%)	117 (36%)	21 (54%)	19 (22%)	126 (45%)
Use of intrauterine device	17 (6%)	54 (16%)	3 (8%)	16 (19%)	35 (12%)
Postmenopausal Use of hormone replacement therapy	20 (7%) 17 (85%)	29 (9%) 24 (83%)	0 0	7 (8%) 5 (71%)	28 (10%) 19 (68%)

Study specimens

Cervical fluid

Before any other sampling or manipulation of the cervix, fluid samples were collected by keeping a Dacron polyester swab in the cervical canal for precisely 20 seconds under visual control. Thereafter, the swab was flushed in 1.5 ml of physiological saline for 2 min. The saline solution was kept frozen (-21 °C) until analyzed (Väisänen-Tommiska *et al*. 2003).

Pap smears

Pap smears were collected at routine screening visits or on a clinical basis if needed. Cells were collected from the vagina, around the internal os of the cervical canal, and inside the cervical canal. Cytological classification was based on the Bethesda 2001 system (Smith 2002a). Low-grade cytological squamous changes covered diagnoses of ASC-US and LSIL. None of the studied women had glandular low-grade atypia. The high-grade category covered "atypical squamous cells – cannot exclude high grade intraepithelial neoplasia" (ASC-H), "atypical glandular cells – favors neoplasia", and HSIL. In Study I Pap smears were evaluated for specific HPV-associated changes, such as parakeratosis, dyskeratosis, angular nuclei, and amplifolia.

Cervical biopsies

Cervical biopsy samples, taken by Schumacher punch biopsy forceps (Stiefel Laboratories, Wooburn Green, Bucks, UK), were collected at the sites which stained positively with acetic acid at colposcopy. The samples were studied microscopically, classified according to CIN classification (Kumar *et al.* 2010) and grouped as normal, CIN 1, and ≥ CIN 2 (Studies II, IV and V). After 1–4 diagnostic biopsy samples two research samples were taken. From women undergoing surgical procedures the biopsy samples were collected under general anesthesia from the squamo-glandular junction at 6 and 12 o`clock positions before possible cervical manipulation (Study IV). One research sample was snap-frozen in liquid nitrogen and stored at -80°C for subsequent Western blotting, and the other one was fixed in formalin, dehydrated and embedded in paraffin for immunohistochemistry.

Detection of infection

High-risk human papillomavirus

Samples taken to the presence of high-risk HPV were collected by inserting a specific brush (DNAPAP Cervical Sampler Hybrid Capture 2, Digene Corporation, Gaithersburg MD, USA, or Roche AMPLICOR HPV test, Molecular Diagnostics, Roche Molecular Systems, Inc., USA) into the cervical canal. The brush was either left at the bottom of the transport tube (Hybrid Capture 2) or removed after flushing into the transport liquid (AMPLICOR). Both samples were stored at room temperature for a maximum of three weeks before analysis.

Both tests detect the same 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) without differentiating between them. Hybrid capture 2 is a semi-automated test involving long single-stranded RNA for hybridization with high-risk HPV DNA in the sample. Thereafter, these RNA-DNA hybrids bind to specific immobilized on microplates.

The AMPLICOR HPV test utilizes the polymerase chain reaction. Initially, the DNA strands are dissociated by heating the sample, then a set of consensus primers hybridizes with an HPV DNA sequence of the conserved region of the L1 gene and initiates polymerization via a heat-stable DNA polymerase. This polymerization is cycled 20–40 times, yielding about 1 billion copies after 30 thermal cycles. Both tests were used in Study II, while in the rest of the studies only the AMPLICOR HPV test was used.

Chlamydia trachomatis

Chlamydia trachomatis was detected by using a commercial test (Gen-Probe Incorporated, San Diego, USA). Cervical samples were collected by rotating a specific swab (Aptima Unisex Swab Specimen Collection Kits, Gen-Probe Incorporated, San Diego, USA) in the cervical canal or by collecting 2 ml of in initial urine stream in a transport tube (Aptima Urine Specimen, Gen-Probe Incorporated, San Diego, USA). The presence of *Chlamydia trachomatis* was assessed by isothermal transcription-mediated amplification of ribosomal RNA. The samples were manipulated to release *Chlamydia trachomatis*-specific RNA, followed by its amplification and formation of an RNA:DNA hybrid which is measurable in a luminometer (Gen-Probe Incorporated, San Diego, USA). This technique is capable of detecting a single copy of *Chlamydia trachomatis* RNA in the sample.

Bacterial vaginosis and candida

Women were judged to have bacterial vaginosis if clue cells were present in Pap smear. Clue cells are cells which are completely covered by coccobacilli. Infection of candida was based on cytological evaluation as well, and women showing typical pseudohyphae or fungal orgnanisms in Pap smear were judged to have candida infection.

Measurement of nitric oxide

Metabolites

Concentrations of NOx were measured spectrophotometrically from the cervical fluid samples. The samples (500 µl) were centrifuged (2200 × g, for 10 min at + 4°C) and thereafter nitrate reduction was carried out by incubating 125 µl of the sample with 5 µl (10 U/ml) nitrate reductase (Boehringer Mannheim), 5 µl (20 mM) nicotinamide adenine dinucleotide phosphate-oxidase (Boehringer Mannheim), 5 µl (1 mM) flavinine adenine dinucleotide (Boehringer Mannheim), and 50 µl phosphate-buffered saline for 15 minutes. To avoid interference of remaining nicotinamide adenine dinucleotide phosphate-oxidase with the chemical detection of nitrite it was removed by incubation with 1.25 µl (3.75 mM) lactate dehydrogenase (Boehringer Mannheim) and 100 µl (15 mM) pyruvate (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes. Total nitrite was then measured by adding Griess reagent to the supernatant. This was prepared by mixing equal volumes of 10% p-aminobenzenesulfoamide (Sigma-Aldrich, St. Louis, MO. USA) in 25% phosphoric acid (Riedel-de Haen AG, Seelze, Germany) and 1% N-(1-naphthyl)-ethylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA) immediately before use (Väisänen-Tommiska *et al.* 2003). The Griess reaction was carried out in duplicate, and absorbance was read at a wavelengths of 546 nm against sodium nitrate (Merck) standards (0, 1.25, 2.5, 5, 12.5, 25 and 50 µmol/l) prepared in water and processed in the same way as the samples. An individual blank was prepared for every sample, and the absorbance obtained from the blank was subtracted from that of the sample. The detection limit of the assay was 0.8 µmol NOx/l of cervical fluid, and intra- and interassay coefficients of variation were 1.6 and 2.4%, respectively (Väisänen-Tommiska *et al.* 2003).

Synthases

Western blotting

Western blot analysis was carried out to assess the expression of eNOS and iNOS proteins in the cervical tissue samples. The frozen biopsy samples were crushed and homogenized with Precellys 24 (Bertin Technologies, Villeurbanne, France) in buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (Sigma-Aldrich, St Louis, MO, USA). The samples were clarified by centrifugation and stored at -140 ºC until used. Total protein concentrations were quantified by using Bio-Rad Protein Assays (Bio-Rad Laboratories, Hercules, CA, USA). Samples containing 25 µg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 150 V for 1 h (3–8% Tris-Acetate SDS-PAGE gel; Invitrogen, Carlsbad, CA, USA) and the proteins were transferred to a polyvinylidene fluoride membrane (pore size 450 µm; 15 V for 35 min) by wet blotting. The membranes were blocked in 3% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) at room temperature for 1h. Primary antibody incubations (mouse monoclonal eNOS/NOS Type III, 1:2500 and iNOS/NOS Type II, 1:10000, BD Transduction Laboratories, Franklin Lakes, NJ, USA) were carried out at +4 ºC overnight. Polyclonal rabbit anti-mouse immunoglobulins coupled to horseradish peroxidase (DAKO, Glostrup, Denmark) were used as secondary antibodies. Specific signals were detected by means of an enhanced chemiluminescence system (ECL Plus, Amersham GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions and visualized by exposure to ECL hyperfilm (Amersham, UK). Signal intensity was quantified by densitometry (Syngene, Synoptics Limited, Cambridge, UK).

Human umbilical vein endothelial cell lysates (Biomedicum Helsinki, Finland) and lysates of interferon-γ/lipopolysaccharide-treated mouse macrophages (Transduction Laboratories, USA) were used as positive controls for eNOS and iNOS, respectively. Actin (C-2, 1:750; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a protein loading control.

Immunohistochemistry

Paraffin sections (5 µm) were deparaffinized and pretreated by heating in a microwave oven in 0.01 M citric acid buffer (pH 6.0) for antigen retrieval. Next, immunohistochemistry was carried out with the help of a Labvision autostainer (LV-1; Thermo Fisher Scientific Inc.,

Fremont, CA, USA) and a PowerVision+^{TM} Poly-HRP IHC Detection System (Leica Biosystems Newcastle Ltd., UK) according to the manufacturers' instructions. The sections were sequentially incubated with 3% hydrogen peroxide, pre-blocking solution, primary antibodies (polyclonal rabbit antibodies for eNOS and iNOS, Thermo Fisher Scientific; 1:400, RT, 60 min), post-blocking solution, and poly-HRP IgG polymer for post-blocking. Tris-buffered saline with Tween, or aqua, was used for washing. Antigens were localized by using diaminobenzidine tetrahydrochloride Plus Substrate. The sections were counterstained with Mayer`s hematoxylin solution (Merck) rinsed with aqua and manually mounted.

Sections of umbilical cord were used as positive controls for both eNOS and iNOS. Negative control measures included replacing primary antibody with rabbit IgG (Negative Control for Rabbit IgG Ab-1, Thermo Fisher Scientific) and incubating slides without primary antibody.

Statistical analyses

Baseline clinical data were analyzed by means of the Chi-square test and Fisher`s exact probability test. The NOx data were expressed as medians with 95% confidence intervals (CIs) and NOS data as mean ± standard error (SE) with 95% CI. Neither NOx nor NOS data were normally distributed and thus significances were tested by means of the Mann–Whitney test or the Kruskall–Wallis test with Bonferroni correction for multiple comparisons. Correlations were assessed by calculating Spearman`s correlation coefficient. Logistic regression analyses were performed to study the prediction of high-risk HPV persistence according to different variables. All tests were two-sided and processed by using SPSS 14 (Chicago, IL, USA) or PASW 18 (IBM Corporation, New York, USA) software. Probability values < 0.05 were considered statistically significant.

RESULTS

The main data are presented here; details are shown in the original publications.

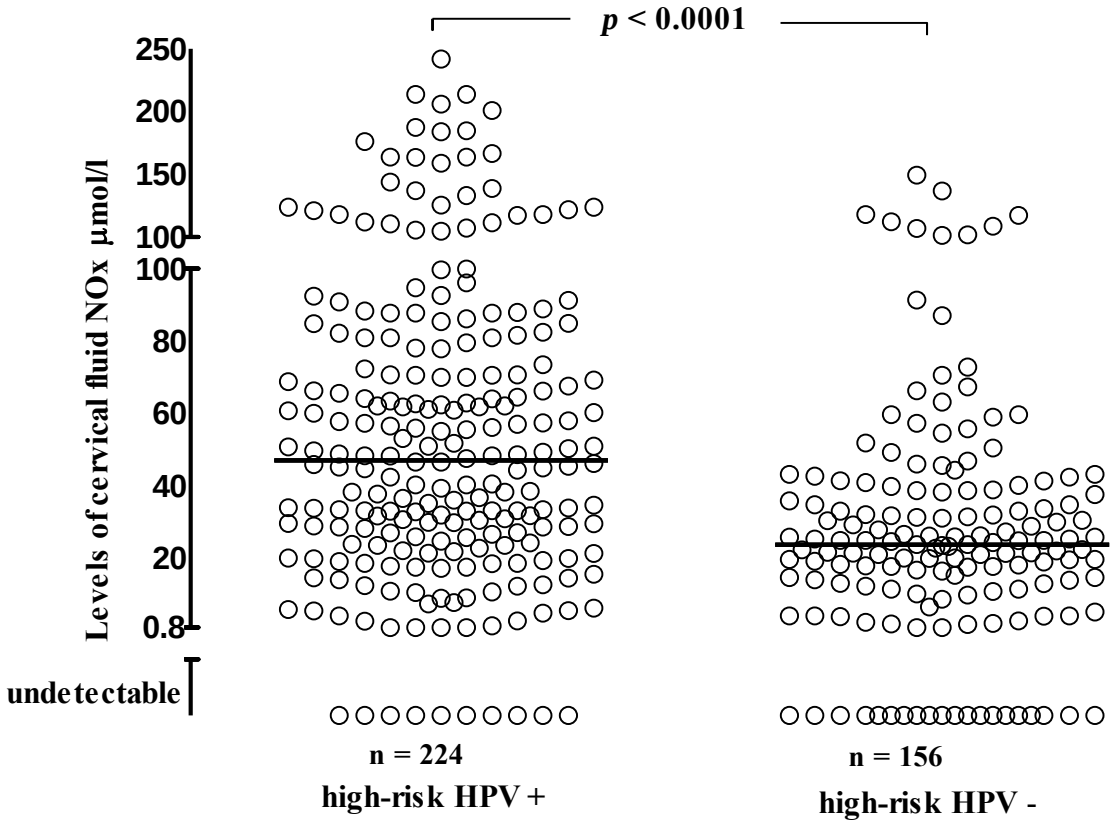
Clinical characteristics

The women with abnormal cytology suggestive of HPV infection were more often nulliparous compared with the women with normal cytology (Study I). Of the women assessed for the presence of high-risk HPV, 290 (58%) were infected and 214 (42%) were non-infected (Studies II–V). Those with high-risk HPV were younger, more often used oral contraceptives, and were more often nulliparous. The women infected with *Chlamydia trachomatis* were younger compared with non-infected women (Study III).

Nitric oxide and human papillomavirus infection

Cervical fluid NOx levels ranged from undetectable to 242 μmol/l. Among women with abnormal cytology suggestive of HPV infection, median NOx levels were 102% higher than in women with normal cytology (22.5 μmol/l, 95% CI: 14.6–31.9 *vs.* 11.0 μmol/l, 95% CI: 8.0–16.7) ($p < 0.001$). The women with high-risk HPV infection (combined data from Studies II and V) showed significantly higher NOx levels (47.1 μmol/l, 95% CI: 38.7–55.6) than the women without such infection (23.8 μmol/l, 95% CI: 20.4–26.7) ($p < 0.0001$) (Figure 5). In subgroup analyses, clinical variables such as parity, use of oral contraceptives and intrauterine devices, did not affect NOx levels (Study II).

Figure 5. Levels of cervical fluid nitric oxide metabolites (NOx) in women with and without high-risk human papillomavirus (HPV) infection (Studies II and V).



Nitric oxide in cytological and histological lesions associated with human papillomavirus infection

In women with normal cytology those with high-risk HPV infection had higher NOx levels (45.2 µmol/l, 95% CI: 31.66–1.6) than women without such infection (21.5 µmol/l, 95% CI: 17.0–26.2) (p = 0.002). High-risk HPV infection was accompanied by elevated NOx levels in women with low-grade cytological changes (57.0 µmol/l, 95% CI: 48.7–63.0 *vs.* 26.0 µmol/l, 95% CI: 17.4–41.3) (p = 0.002). In women with CIN 1, those infected with high-risk HPV had higher NOx levels (48.8 µmol/l, 95% CI: 42.95–6.7) compared with non-infected women (23.4 µmol/l, 95% CI: 23.6–41.2) (p = 0.003) (Figures 6,7, Studies II and V). Women with high-risk HPV infection and low-grade cytological changes had higher NOx levels (57.0

µmol/l, 95% CI: 48.7–63.0) than those with high-grade changes (28.8 µmol/l, 95% CI: 1.2–70.4) ($p = 0.001$) (Figure 6, Studies II and V).

Figure 6. Levels of cervical fluid nitric oxide metabolites (NOx) in women with and without high-risk human papillomavirus (HPV) infection and with normal or abnormal cervical cytology (Studies II and V).

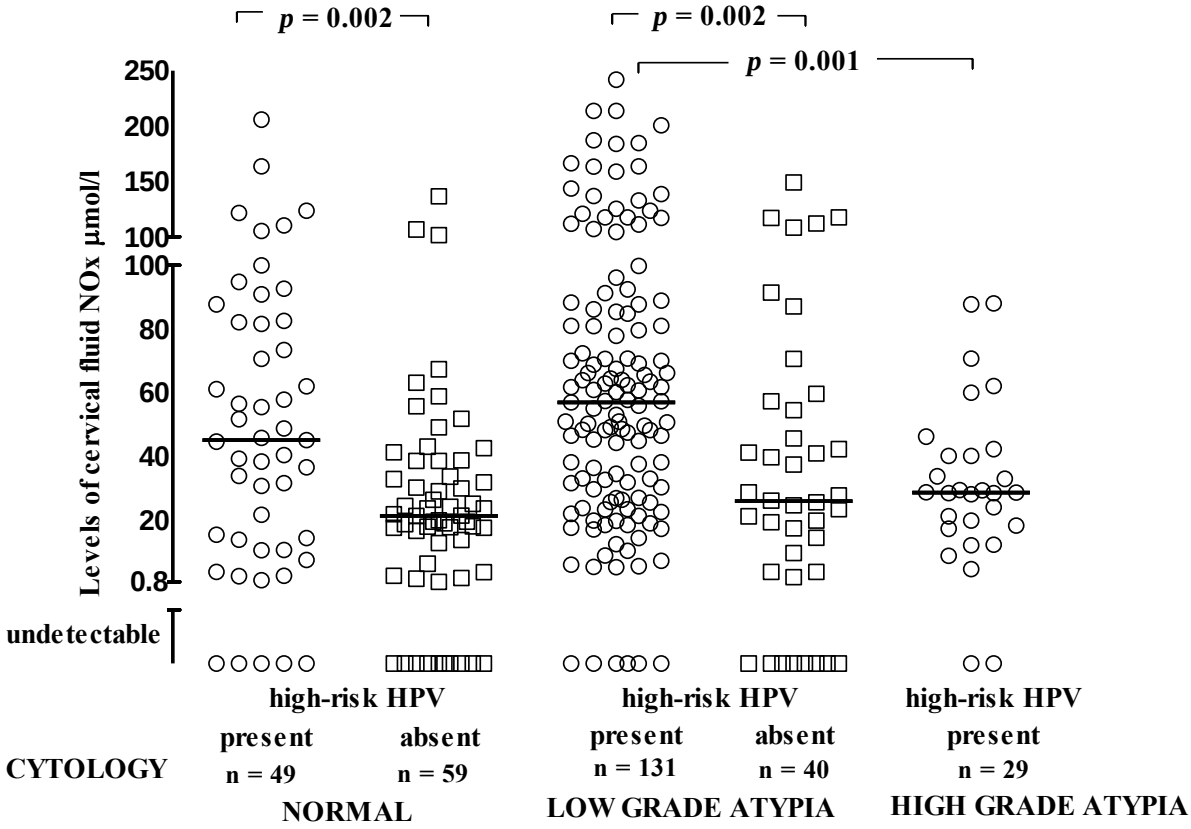
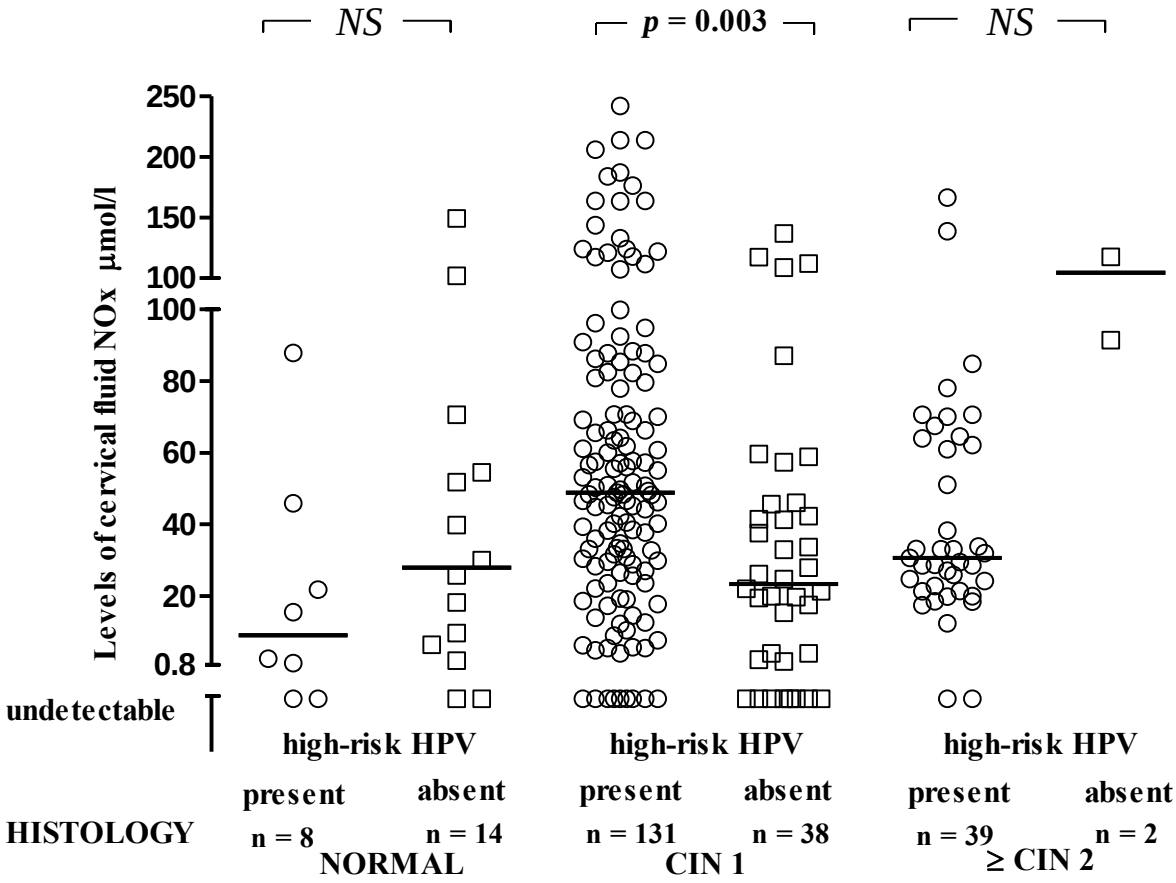


Figure 7. Levels of cervical fluid nitric oxide metabolites (NOx) in women with and without high-risk human papillomavirus (HPV) infection and with normal or abnormal cervical histology (Studies II and V). *NS* = non-significant.



Nitric oxide metabolites and other relevant gynecological infections

Women infected with *Chlamydia trachomatis* had higher NOx levels (37.5 µmol/l, 95% CI: 6.1–50.9) than women without such infection (19.7 µmol/l, 95% CI: 5.6–30.0) (p = 0.02) (Study III). Neither bacterial vaginosis nor candida infection had an effect on cervical fluid NOx levels, but the number of studied women with these infections was limited (Table 4, Study II).

Table 4. Levels of cervical fluid nitric oxide metabolites [NOx median μmol/l (95% CI)], in women with (+) and without (-) high-risk human papillomavirus (HPV) and *Chlamydia trachomatis*, bacterial vaginosis, and candida infections.

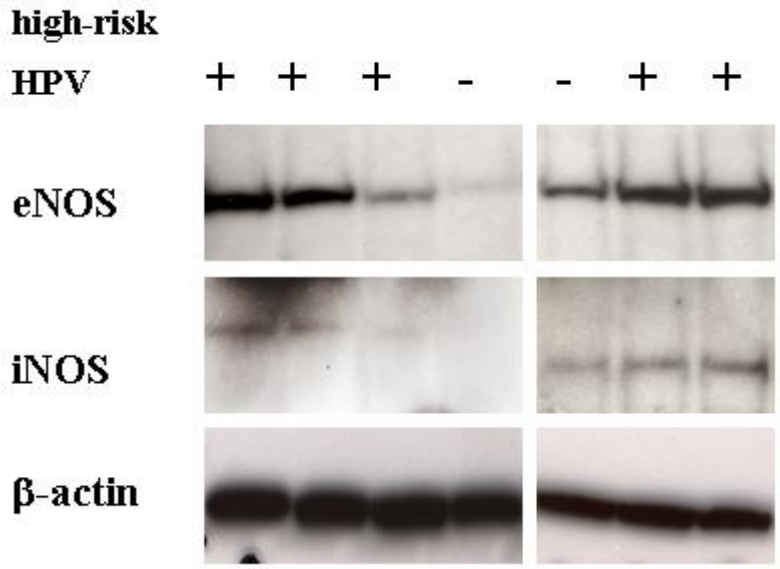
Subgroups	**high-risk HPV +**		**high-risk HPV -**		***p***
	NOx	**n**	**NOx**	**n**	
Chlamydia trachomatis +	27.2 (17.1-50.9)	14	50.9[1]	5	0.05
Chlamydia trachomatis -	36	3	18.7[1] (2.0-28.6)	15	0.2
Bacterial vaginosis +	36.2 (24.3-62.9)	20	27.3 (10.1–54.6)	19	0.2
Bacterial vaginosis -	48.7 (36.5–56.6)	139	22.8 (18.3–26.2)	79	< 0.001
Candida +	47.6 (21.4–60.8)	16	41.3[2] (12.8–54.6)	9	0.3
Candida -	44.9 (33.9–55.7)	159	23.7[2] (20.9–25.4)	144	< 0.001

[1]$p = 0.02$, [2]$p = 0.2$

Nitric oxide synthase and high-risk human papillomavirus infection

High-risk HPV infection was associated with elevated expression of both eNOS and iNOS proteins in Western blot analysis (eNOS: mean 33.8 ± 5.6, 95% CI: 22.5–45.1 *vs.* 20.2 ± 6.8, 95% CI: 6.1–34.3, $p = 0.007$; iNOS: mean 12.0 ± 2.4, 95% CI: 7.1–16.9 *vs.* 5.6 ± 1.8, 95% CI: 2.0–9.2, $p = 0.003$) (Figure 8, Study IV). Positive controls for eNOS (human umbilical vein endothelial cell lysates) and iNOS (lysates of interferon-γ / lipopolysaccharide-treated mouse macrophages) showed bands in Western blots as expected.

Figure 8. Western blotting of endothelial (e) and inducible (i) nitric oxide synthase (NOS) protein expression in cervical samples from women with (+) and without (-) high-risk human papillomavirus (HPV) infection.



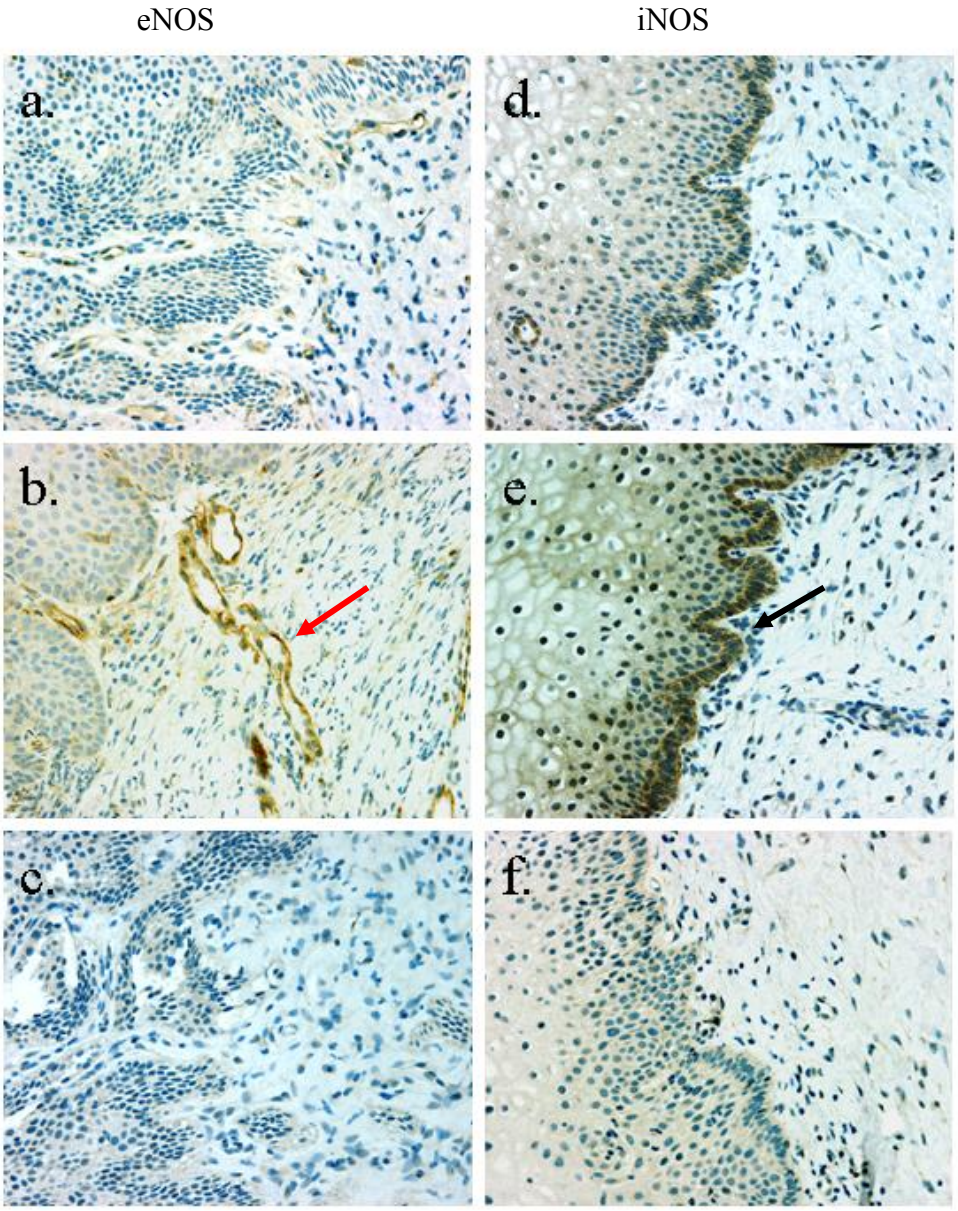
Thirty-two cervical biopsy samples were randomly selected for immunohistochemistry (16 with high-risk HPV, 16 without high-risk HPV), in which eNOS was localized in vascular endothelium while iNOS was detected typically in basal squamous epithelial cells (Table 5). Inflammatory cells were detected in nine women with high-risk HPV and in three women without such an infection. None of inflammatory cells in high-risk HPV-negative samples stained for iNOS, whereas three high-risk HPV-positive samples stained for it (Table 5, Study IV). In positive controls eNOS was localized in vascular epithelium and iNOS in smooth muscle and some inflammatory cells, whereas no immunostaining for either eNOS or iNOS was seen in the negative controls (Figure 9).

Table 5. Immunohistochemical staining intensity of inducible nitric oxide synthase (iNOS) in squamous epithelial and inflammatory cells from 32 women with (+) (n = 16) and without (-) (n = 16) high-risk human papillomavirus (HPV) infection. The numbers indicate in how many samples staining was detected. One sample could show staining in different layers of epithelium and/or inflammatory cells. (Study IV).

	Squamous epithelial cells										**Inflammatory cells**	
	basal				**parabasal**		**suprabasal**		**immature metaplasia**			
iNOS	**(+)**	**+**	**++**	**+++**	**(+)**	**+**	**+**	**++**	**+**	**++**	**visible**	**iNOS**
high-risk HPV + (n = 16)	3	2	11		4	1	2	1	1	2	9	3
high-risk HPV- (n = 16)	2	6	1	1					2		3	

(+) = very weak staining, + = weak staining, ++ = moderate staining, +++ = intense staining

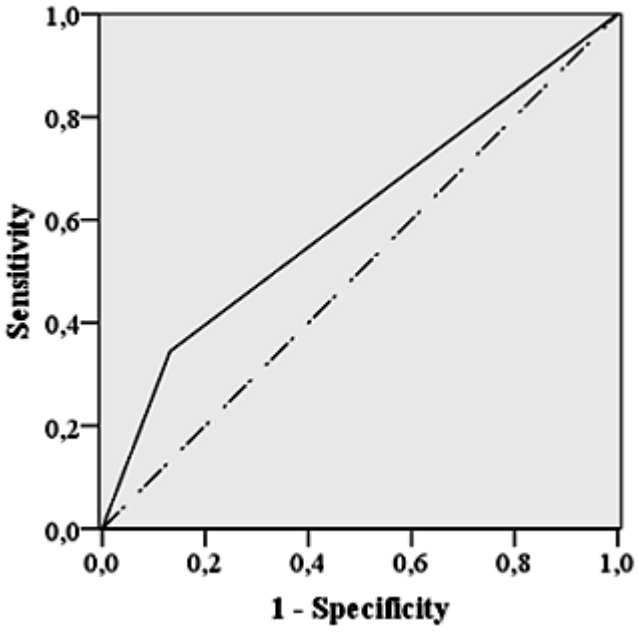
Figure 9: Immunohistochemical localization of endothelial nitric oxide synthase (eNOS) (left panels, a–c) and inducible nitric oxide synthase (iNOS) (right panels, d–f) in cervical samples from women without (a, d) and with (b, e) high-risk human papillomavirus infection. Endothelial NOS was localized in the vascular endothelium (black arrow) and iNOS mainly in the basal layer of epithelial cells (red arrow). Specimens incubated with irrelevant antibody (IgG) showed no immunoreactivity (c, f). Hematoxylin counterstain, × 200 magnification. (Study IV).



Nitric oxide and persistence of high-risk human papillomavirus infection

High-risk HPV-infected women were followed for 12 months and re-tested for the presence of high-risk HPV (Study V). The women with cervical lesions with CIN indicative for Loop conization were excluded from predictive analysis. The women with persistent infection had higher baseline NOx levels (56.9 µmol/l, 95% CI: 48.7–81.0) compared with those with eradicated high-risk HPV (37.7 µmol/l, 95% CI: 27.0–58.0) (p = 0.02). Regarding baseline NOx quartiles, the 75th percentile (87 µmol/l) significantly discriminated women with detectable and undetectable high-risk HPV at 12 months (p = 0.04). This cut-off value predicted high-risk HPV persistence with an OR of 4.1 (p = 0.02) and showed 33% sensitivity, 90% specificity, an 85% positive predictive value, a 44% negative predictive value, a 10% false-positive rate, and a 67% false-negative rate. A receiver operating characteristic curve (solid line) dividing true-positive (sensitivity, y-axis) and false-positive (1-specificity, x-axis) results gave an area of 0.62 (95% CI: 0.52–0.69) (p = 0.02) for this cut-off value (87 µmol/l) in predicting high-risk HPV persistence (Figure 10).

Figure 10. Receiver operating characteristic curve for baseline cervical fluid nitric oxide metabolites: 87 µmol/l in differentiating the presence and absence of high-risk human papillomavirus after 12 months of follow-up. Broken line = reference line.



DISCUSSION

Although the causality of high-risk HPV in cervical cancer is one of the strongest in epidemiology (Bosch *et al*. 2008), fortunately most HPV infections are cleared spontaneously (Moscicki *et al*. 2004, Stanley 2010b, Woo *et al*. 2010). However, the mechanisms leading to dysplasia and ultimately to cancer cannot be recognized and thus mass screening by means of Pap smears and/or HPV tests, and follow-up are needed. Due to the commonness of HPV infection this is laborious and expensive and furthermore may cause unnecessary anxiety in women with a transient infection (Maissi *et al*. 2005). Thus, more information on the course of high-risk HPV infection and reliable biomarkers identifying the women with persistent high-risk HPV infection are needed. Nitric oxide is a very potent biomediator, and therefore, its relationships to HPV infection were studied in this work.

Study population and methods: strengths and limitations

The majority of the women in this study (58%) had high-risk HPV infection, most likely due to the fact that many of them had a history of abnormal cervical cytology. As expected, women with high-risk HPV were younger than non-infected ones, although more elderly women were also included, since another incidence peak of HPV infection is at the age of menopause (Burchell *et al*. 2006). The aim was to study nitric oxide during the natural course of HPV infection and thus, women who had undergone Loop conization were excluded from the predictive analyses, since it is known that conization is associated with eradication of high-risk HPV (Kim *et al*. 2009, Nam *et al.* 2009). Pregnant women were also excluded, because cervical nitric oxide release is elevated during pregnancy (Väisänen-Tommiska 2006a). Moreover, women with any cervical bleeding were excluded, since it is known that the presence of blood in the sample reduces NOx levels because hemoglobin binds to nitric oxide and forms a nitrosylhemoglobin complex (Kankaanranta, *et al*. 1996). No power analyses were carried out in the beginning of the study, since no previous data on the levels of cervical fluid NOx in non-pregnant women existed. Although the total number of women

tested for the presence of high-risk HPV was high, some subgroups were small and thus the data should be interpreted with caution.

Nitric oxide is converted rapidly to nitrite and nitrate, the levels of which indicate the release of nitric oxide in various biological fluids (Miranda *et al.* 2001). These levels were measured by means of a validated method used in several previous studies carried out in our laboratory (Ranta, *et al.* 1999, Väisänen-Tommiska 2006a) and elsewhere (Shaamash *et al*. 2005). Cervical fluid NOx levels do not correlate with plasma NOx levels and are not affected by dietary intake of nitrites-containing food (Väisänen-Tommiska 2006a). Thus, no dietary restrictions were needed in the women studied. The levels of NOx varied greatly and thus it is possible that larger sample sizes would have been needed for additional conclusions. The study subjects were not routinely tested for the presence of other cervicovaginal infections, although women with clinical signs of infection were excluded. Moreover, the production of nitric oxide may change during the course of HPV infection, but it was not possible to determine the time at which HPV had been acquired. However, HPV typically first triggers low risk cytological changes, followed by different grades of histological changes and thus comparison of NOx levels in cytological and histological samples enables some estimation of the stage of high-risk HPV infection.

Two different study populations were used in the present work. One was collected from a mass-screening program and the other from a colposcopic unit to which women had been referred on the basis of previous Pap smear findings. Levels of cervical fluid NOx differed in these groups, presumably because women with history of Pap smear changes probably had had HPV infections. However, comparison of HPV-infected and non-infected women was carried within each study group and thus this variation does not invalidate the conclusions.

In addition to NOx assessment the mechanisms behind cervical nitric oxide release were eluciated by assessing eNOS and iNOS by both Western blotting and immunohistochemistry. Concomitant use of these methods enables quantitative assessment of eNOS and iNOS expression and also identification of the location of these activities in cervical cells. To best of my knowledge this study is the first one involving Western blots in association with nitric oxide and HPV, whereas previous studies have solely involved the use of semi-quantitative immunohistochemistry (Hiraku *et al.* 2007, Mazibrada *et al.* 2008) (Table 6). I acknowledge that the high-risk HPV tests employed could detect only 13 papillomavirus types, although approximately 40 other HPV types may infect the genital mucosa (Bosch *et al.* 2003). Thus, women infected with HPV types other than the 13 selected ones tested negatively in the

present work. Nevertheless, these 13 types of high-risk HPV cover most of the HVP genotypes detected in cervical cancer (Bosch *et al.* 2003). The tests applied do not allow differentiation of high-risk HPV genotypes and therefore, in the follow-up study it was not possible to determine if some women were free of the initial infection but had acquired a new one. Moreover, two different HPV tests were employed in Study II, but their correlation is between 83–89.2 % (Sandri *et al*. 2006, Carozzi *et al.* 2007).

Principal findings on human papillomavirus infection

Women with cytological changes suggestive of HPV infection had higher NOx levels compared with women with normal cytology. This result was confirmed by the finding that women discovered to have high-risk HPV showed elevated cervical NOx levels. Furthermore, high-risk HPV infection was associated with elevated expression of both eNOS and iNOS proteins. Previous data on HPV and nitric oxide are relatively scant (Table 6). The only *in vivo* study did not show any difference in iNOS immunostaining between various high-risk HPV genotypes (Mazibrada *et al*. 2007). Moreover, HPV seems to suppress the activity of the tumor suppressor protein p53 (Wei *et al.* 2009). In addition, the transduction of HPV 16 oncoproteins E6 and E7 stimulates iNOS (De Andrea *et al*. 2005). Thus, these findings give further support for the claim that high-risk HPV is capable of directly inducing nitric oxide release. This is supported by previous immunohistochemical data (Hiraku *et al.* 2007, Mazibrada *et al*. 2008) and by my own *in vivo* findings, which were in accordance with these data. Further support for a direct connection between HPV and nitric oxide may be gained from the fact the iNOS expression is strongest in basal squamous cells, which are first attacked by HPV. On the other hand, it is possible that high-risk HPV induced cytokines, such as interferon-γ and tumor necrosis factor-α contribute to nitric oxide release (De Andrea *et al*. 2007, Song *et al*. 2008). This is also supported by the present results showing that high-risk HPV induced eNOS expression in vascular endothelium, which is not a target of HPV. Perhaps this is mediated through vascular endothelial growth factor (Song *et al.* 2006, Kuemmel *et al.* 2009, Monk *et al*. 2010). Furthermore, in the present study inflammatory cells, although few in number, showed some iNOS expression. However, this was so little that probably high-risk HPV was responsible for iNOS activation.

The role of enhanced cervical nitric oxide release during high-risk HPV infection is not clear. Nitric oxide inhibits viral replication *in vitro* (Reiss *et al*. 1998, Akaike *et al*. 2000) and thus, in the human uterine cervix elevated nitric oxide levels may promote high-risk HPV eradication. This is supported by *in vitro* data showing that macrophages need nitric oxide to kill HPV 16 E6 and E7 oncoprotein-expressing cells (Routes *et al*. 2005). On the other hand, cervical nitric oxide could be related to HPV oncogenes E6 and E7, which are dose-dependently upregulated by nitric oxide (Wei *et al*. 2009). These oncogenes downregulate host tumor suppressor proteins, such as p53 (Hebner *et al.* 2006), which normally participate in regulation of the cell cycle (Hussain *et al.* 2006). In addition, it is known that high nitric oxide levels may damage DNA (Lala *et al.* 2001). Thus, HPV, by causing nitric oxide release and by suppressing p53 may arrest normal cell repair and thus pave the way to the development of cancer. It is also possible that nitric oxide has a dual effect in high-risk HPV infection and it initially takes part in HPV clearing, but if this fails, nitric oxide may become a promoter of disease progression.

Table 6a. Previous *in vitro* studies on cervical and vaginal nitric oxide, human papillomavirus (HPV) and cervical dysplasia. iNOS = inducible nitric oxide synthase, CIN = cervical intraepithelial neoplasia.

Study	Method and study setting	Main results
in vitro studies		
De Andrea *et al.* 2007	- Expression of iNOS messenger RNA and nitrate/nitrite levels - Normal human keratinocytes and an immortalized cell line - HPV 16 E6 and E7 oncoproteins	- HPV 16 oncoproteins E6 and E7 increase iNOS messeger RNA and nitrate/nitrite levels both in normal and immortalized cell lines
Wei *et al.* 2009	- HPV gene transcription after exposure to nitric oxide - Tumor suppressor protein p53 levels after exposure to nitric oxide - Cells from CIN (HPV 16, HPV 31), HPV-negative cervical carcinoma cells, keratinocytes and immortalized cells with normal and mutant p53 proteins	- High nitric oxide concentration induces HPV gene (E1, E4, E6, E7) RNAs dose-dependently - High nitric oxide concentrations decrease tumor suppressor protein p53 levels and activity in HPV-infected cells - High nitric oxide concentrations induce DNA double strand breaks and mutations in HPV-positive cells

Table 6b. Previous *in vivo* studies on cervical and vaginal nitric oxide, human papillomavirus (HPV) and cervical dysplasia. iNOS = inducible nitric oxide synthase, CIN = cervical intraepithelial neoplasia.

Study	Method and study setting	Main results
in vivo studies		
Hiraku *et al*. 2007	- Immunohistochemical staining of iNOS - Women with condyloma acuminatum and different grades of CIN	- iNOS localization in cytoplasm of epithelial cells and stromal inflammatory cells - iNOS in stroma higher in CIN *vs* condyloma acuminatum - iNOS in stroma positively correlated with CIN grade
Tavares-Murta *et al.* 2008	- Nitric oxide metabolites in cervical and vaginal fluid (Griess reaction) - Women with and without bacterial vaginosis, and different grades of CIN	- Nitric oxide metabolites higher in cervical fluid *vs* vaginal secretion - Nitric oxide metabolites higher in women with CIN *vs*. bacterial vaginosis both in cervix and vagina
Mazibrada *et al.* 2008	- Immunohistochemical staining of iNOS - Women with normal histology, different grades of CIN, or with cervical squamous cancer	- No difference in iNOS immunostaining between different high-risk HPV genotypes - iNOS localization in cytoplasm of squamous epithelial cells in basal and parabasal layers, and inflammatory cells - Highest iNOS immunostaining in women with CIN1 - iNOS immunostaining significantly reduces as the progression of dysplasia increases
da Silva *et al.* 2010	- Immunohistochemical staining of iNOS - Women with normal histology, CIN 3, or with invasive cervical cancer	- No difference in iNOS immunostaining between normal histology and CIN 3 - iNOS immunostaining stronger in the stroma of peritumoral region compared with normal histology

Other relevant gynecological infections

To evaluate nitric oxide release in other cervical and vaginal infections I compared NOx levels in women with *Chlamydia trachomatis*, bacterial vaginosis, and candida with and without simultaneous high-risk HPV infection. Neither bacterial vaginosis nor candida showed any effect on cervical NOx levels. The data on NOx and candida are novel, but one previous study has shown normal levels of NOx in cervical fluid of women with bacterial vaginosis (Tavares-Murta *et al*. 2008). Larger study populations are needed to reveal if significant differences truly exist.

In contrast, *Chlamydia trachomatis* infection was associated with elevated NOx levels. Both iNOS and nitric oxide are related to *Chlamydia trachomatis*, at least in cell and animal experiments. The significance of nitric oxide in connection with *Chlamydia trachomatis* is unknown but it may be a factor in the clearance of infection, or complications; the data are far from being uniform in this regard (Chen *et al*. 1996, Ramsey *et al.* 2001a, Ramsey *et al*. 2001b, Brunham *et al.* 2005, Roshick *et al.* 2006, Shao *et al*. 2010). Moreover, it should be emphasized that *Chlamydia trachomatis* is an independent risk factor of squamous cervical cancer (Paavonen *et al*. 1999, Anttila *et al*. 2001, Smith *et al*. 2002b) and thus, it is possible that *Chlamydia trachomatis*-mediated nitric oxide may be involved.

Cervical lesions and nitric oxide

Women with low grade cytological changes showed higher cervical NOx levels compared with women with high grade changes. In addition, both eNOS and iNOS expression levels were found to be elevated in women with histological cervical lesions. These findings are in line with those of other studies (Hiraku *et al.* 2007, Mazibrada *et al.* 2008).

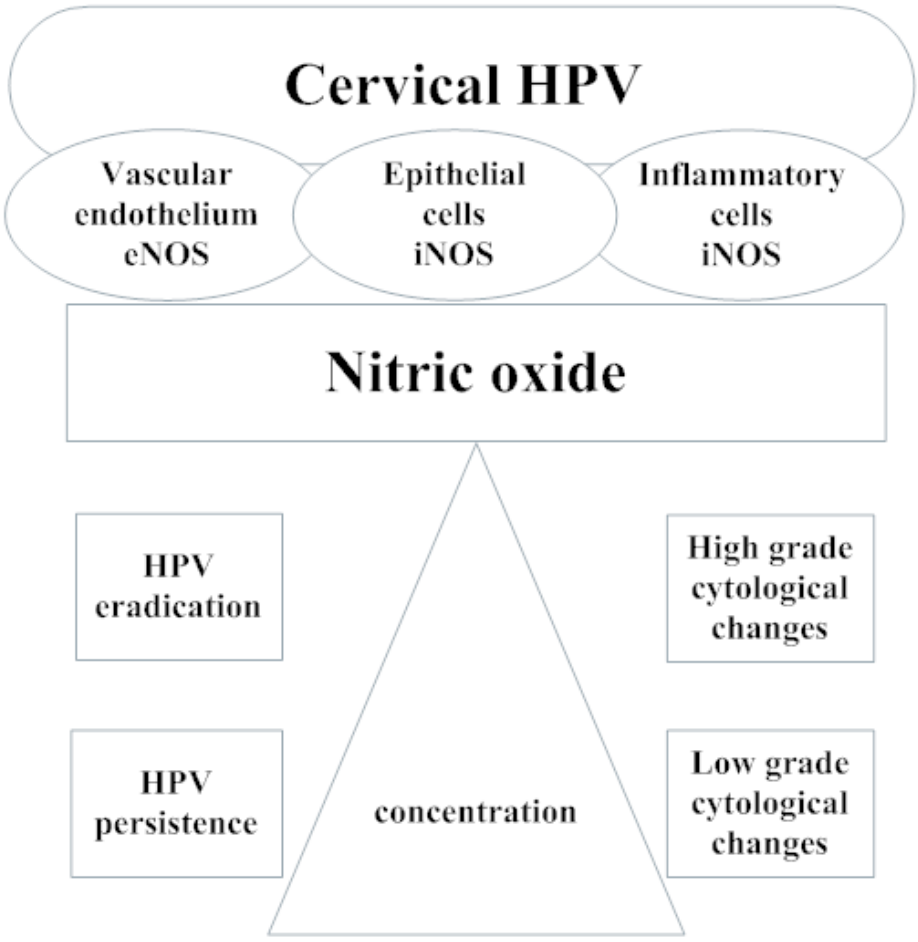
Nitric oxide may have a dual role in immunity and carcinogenesis depending mainly on its concentration; high levels may stimulate immune defense and arrest carcinogenesis whereas low levels have an opposite effect (Ridnour *et al.* 2006). Therefore, it is possible that cases of mild dysplasia, which are most often cleared spontaneously (Wright *et al.* 2003), do so with the aid of nitric oxide release. In contrast, high-grade dysplasia may progress to cancer

perhaps partly because of low nitric oxide release that may result in failed apoptosis (Heller 2008). The availability of nitric oxide in the cervix may also depend on the duration of HPV infection; in the early phase squamous epithelial cells are the primary source of nitric oxide (Mazibrada *et al.* 2008), but in later phases with high grade dysplasia inflammatory and stromal cells take over nitric oxide production (Hiraku *et al.* 2007, Mazibrada *et al*. 2008, da Silva *et al.* 2010). This may be an additional explanation for high NOx levels in connection with mild lesions.

Clinical implications

No reliable biomarkers for high-risk HPV persistence or regression exist. The present data indicate that high baseline cervical NOx levels favor high-risk HPV persistence (Study V), though the clinical usefulness of NOx testing is restricted as a result of its low sensitivity (33%) and high false-positive rate (67%). However, the data indicate that nitric oxide is a mediator that reflects the course of high-risk HPV infection and thus nitric oxide is a potential target for future studies. It would be particularly interesting to compare nitric oxide levels among various high-risk HPV genotypes, since cancerogenic properties of various HPV strains vary (Ramanakumar *et al.* 2010, Schiffman *et al.* 2011, Stoler *et al.* 2011). Initial studies could perhaps be conducted in cell-line experiments. Moreover, connections between cervical nitric oxide and tumor biomarkers, such as p16 and Ki-67 (Kruse *et al.* 2004, Tsoumpou *et al.* 2009) should be explored. Finally, longitudinal large-scale studies with repeated nitric oxide and high-risk HPV sampling are mandatory to assess the prognostic value of cervical nitric oxide in the follow-up of HPV-infected women.

Figure 11. A schematic summary of the findings in the present study.
eNOS = enodothelial nitric oxide synthase, iNOS = inducible nitric oxide synthase.



CONCLUSIONS

On the basis of the present work the following conclusions can be drawn:

1. Typical HPV-induced cytological abnormalities are accompanied by elevated levels of cervical fluid nitric oxide.
2. Cervical nitric oxide metabolite levels and thus the release of nitric oxide are elevated in high-risk human papillomavirus-infected women.
3. Low-grade cytological changes are accompanied by higher cervical nitric oxide metabolite levels compared with high-grade cell abnormalities.
4. *Chlamydia trachomatis* infection is associated with elevated cervical fluid nitric oxide levels.
5. In addition to inducible nitric oxide synthase, high-risk human papillomavirus is associated with elevated levels of endothelial nitric oxide synthase.
6. High levels of cervical nitric oxide metabolites may predict the persistence of high-risk human papillomavirus infection for at least 12 months.

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